AN INVESTIGATION OF CELL CYCLE EVENTS MODULATED BY A SIALOGLYCOPEPTIDE INHIBITOR

by

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TABLE OF CONTENTS

INTRODUCTION

The regulation of cell proliferation is a complex process involving a network of interactions between positive and negative regulatory substances. These substances include nutrients, growth hormones, growth factors and growth inhibitory molecules. However, the exact nature and importance of these molecules in cellular development, differentiation, tumorigenesis, and other biological processes is presently not clear. Therefore, one of the basic challenges in the study of growth regulation is the determination of the biochemical nature and site of action of these molecules, which can shift cells from a proliferative stage to a non-proliferative stage or vica-versa.

Two of these subgroups, growth factors and growth inhibitors, are of particular interest in the study of cell growth regulation. Growth factors are those factors which contribute to the stimulation of macromolecular synthesis and subsequent cell division. These factors are small polypeptides released by cellsand, therefore, their isolation and purification has not created problems for investigators. Because of the convenience of their isolation, the mechanism of action of a multitude of growth factors such as epidermal growth factor (EGF), nerve growth factor (NGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) etc., has been extensively studied.

Growth inhibitors are the second group of factors that are significantly important in the study of cell growth regulation. However, due to the complexities involved in their isolation and purification, the study of their mechanism of action has been quite limited. Despite the obstacles involved in the purification of these molecules, growth inhibitors from both mouse and bovine cerebral cortex cell surfaces have been isolated and purified to homogeneity by members of our laboratory. This dissertation involves study of the effect of a sialoglycopeptide inhibitor, obtained from bovine cerebral cortex cells, on cellular DNA synthesis and cell division. In addition,

the host range for this inhibitory molecule, the exact point in the cell cycle where the inhibitor exerts it's biological activity, and it's effect on the expression of genes known to be involved in cell growth regulation are presented.

CHAPTER I.
HISTORICAL REVIEW.

OVERVIEW

A number of investigators have reported the role of cell surface components in inhibiting cell division. The first experimental results suggesting the existence of cell growth inhibitors were provided by the studies on epidermal cell culture wound healing. In these experiments, Dulbecco and Stocker (1970) demonstrated two findings: 1) the re-initiation of DNA synthesis in "spent" media mainly near the edge of the culture wound and 2) the inhibition of cell division upon confluency. These findings suggested that cell surface components have a possible role in inhibiting cell division. Furthermore, Wittenburger and Glaser (1977) demonstrated that the addition of isolated plasma membranes from growth arrested 3T3 cells to sparse 3T3 cells reversibly inhibited DNA synthesis. This provided further evidence that cell-cell contact is important in growth regulation.

On the other hand, Dulbecco (1970) observed that the initiation of DNA synthesis was also dependent upon the serum level used in the culture medium. This suggested that there were factors in the serum that were also important in cell growth regulation. However, Stocker (1973) demonstrated that "spent" medium was capable of supporting the growth of subconfluence cultures to saturation densities. Stocker's findings implied that the culture medium was not totally depleted of essential nutrients. These results proposed that the cessation of cell proliferation was mediated by either soluble growth inhibitors or through cell-cell contact.

Even though the existence of inhibitors was evident, investigators were faced with a major predicament when experimentally studying growth inhibitors. This difficulty was due to the complexity of membrane component isolation and purification. If not released by cells, these growth inhibitory molecules are resident cell membrane molecules and, therefore, hydrophobic in nature. Due to the hydrophobicity of these molecules, detergents must be utilized for their proper

solubilization. Once these molecules are in detergent, the study of their biological activity and their introduction to living cells was a difficult barrier.

Despite the obstacles involved in the purification of these molecules, there have been numerous reports concerning the presence of growth inhibitors. For example, Bullogh (1965) reported a class of growth inhibitory molecules termed "chalone". Chalones were defined as growth inhibitory molecules which were tissue specific, species non-specific and their action was reversible. Chalones were later found in a variety of tissues including erythrocytes, arterial smooth muscle, liver, kidney, and lung (Bullough 1975). Nonetheless, no chalones have been purified to homogeneity, thus limiting studies concerning such molecules.

Several other investigators have reported on chalone-like molecules isolated from different cells and tissues. A low molecular weight cell growth inhibitor produced by 3T3 cells was briefly reported by Yeh and Fischer (1969). Lipkin and Knecht (1974) reported the isolation of a 160 kD molecular weight melanocyte contact inhibitory factor (MCIF) from the conditioned media of hamster melanocytes. MCIF was capable of restoring contact inhibition to highly malignant melanocytes as well as inhibiting DNA synthesis and cell division in a variety of cell types in vitro. Another inhibitor, isolated from media conditioned by BSC-1 (African Green Monkey kidney epithelial-like) cells (Holley et al., 1978), has been partially purified and termed Growth Inhibitor (GI). The partially purified material contained a 25 kD molecular weight inhibitory protein consisting of two 12.5 kD subunits. This factor was capable of inhibiting DNA synthesis in BSC-1 cells by 85% and reversibly inhibited the actual proliferation of BSC-1 cells. The inhibitory effects of the GI were counteracted by the addition of EGF and calf serum (Holley et al., 1980). GI has inhibitory effects on mouse mammary epithelial, secondary rat lung, and human carcinoma cells in vivo, but had no inhibitory activity against fibroblast cells (Holley et al., 1980; Walsh-Reiz et

al., 1984). Hepatic proliferation inhibitor (HPI) was another inhibitor isolated from liver tissue and was partially purified (McMahon et al., 1982). Upon purification, HPI had a molecular weight of 26 kD and a pI of 4.6, and reversibly inhibited colony formation by non-malignant hepatic cells. Even though work on HPI was quite promising, no further information was released from the laboratories investigating this inhibitor. There also are several reports on the antiproliferative activity of interferons. Mice inoculated with tumor cells showed a slower rate of tumor growth and increased survival if they were simultaneously given mouse interferon (Gresser, 1972; Gresser and Tovey, 1978; Balkwill, 1979; Taylor-Papadimitriou, 1980). Furthermore, tumor necrosis factor (TNF) a monocyte/macrophage-derived protein was found to be cytotoxic to many tumor cells in vitro (Fransen et al., 1986). Most of the reports concerning inhibitory substances only refer to indirect observations or to factors that neither have been structurally identified nor studied with regard to their molecular mechanism(s) of biological activity.

In order to understand cell growth regulation, however, it is of extreme importance to purify growth inhibitors and study their action at a molecular level. To our knowledge, only five genuine growth inhibitors have been isolated and purified to apparent homogeneity, and to a certain degree, characterized. These inhibitors include: a growth inhibitor isolated from mammary glands designated as mammary drived growth inhibitor (MDGI) reported by Bohmer et al. (1984); fibroblast growth regulator-secreted (FGR-s), isolated from density-inhibited 3T3 cultures by Steck et al. (1982); Type-β transforming growth factor (TGF-β) which is structurally similar to GI, isolated from virally transformed cells in culture (Tucker et al., 1984, James and Bradshaw, 1984); and lastly, the glycopeptide inhibitors isolated from intact mouse and bovine brain cerebral cortex cells, described by the members of our laboratory (Kinders et al., 1979; Kinders and Johnson 1982; Sharifi et al., 1986). As more is known about

these molecules, has become clear that some may be related to others. For example, it has been reported that MDGI is functionally and structurally similar to FGR-s and that TGF-β and GI also are very similar. It is possible that inhibitory substances, which at this time seem unrelated, may be combined in the future. The remainder of this literature review focused on the properties of these five inhibitors.

FIBROBLAST GROWTH REGULATOR

Fibroblast growth regulator secreted (FGR-s) is a growth inhibitory molecule which reversibly inhibits DNA synthesis of subconfluent mouse 3T3 cultures and is also capable of reducing the number of 3T3 cells per colony by about 70% (Steck et al. 1982). FGR-s was first isolated by Steck et al. (1979) from media conditioned by dense cultures of 3T3 cells. Although the mechanisms involved in this phenomenon are still not completely clear, Wang and Steck (1982) suggested that inhibitory factors released into the medium by the 3T3 cells themselves may be responsible for at least part of the inhibition of cell division.

When partially purified, FGR-s consisted of two polypeptide chains with molecular weights of 10 kD and 13kD. These molecules were shown to bind to about 3-4 x 10^5 receptors per cell on 3T3 cells surfaces (Steck et al., 1979, 1982). Voss et al. (1982) suggested that this binding was important for biological activity of FGR-s. Their suggestion was based on the fact that binding of FGR-s to 3T3 cells is reduced when the inhibitory action of FGR-s is blocked by serum. Further analysis of the specific binding of FGR-s to target 3T3 cells revealed that the greatest binding is achieved after only 3 h of incubation at 37C (Steck et al., 1982).

Due to the lack of a purified preparation, it was unclear whether one or both polypeptides observed in the FGR-s fraction were responsible for the growth inhibitory activity. One approach to the resolution of this problem was to raise monoclonal antibodies that would bind and/or neutralize the growth inhibitory activity. A rat monoclonal antibody, designated 2A4, was generated which was specific for the 13 kD subunit of the FGR-s. The biological activity of FGR-s was depleted when the total material was passed through an affinity column coated with the 2A4 antibody. It was also shown that antibody 2A4 was capable of neutralizing the growth inhibitory effect of the 13 kD subunit of the FGR-s in a concentration-dependent fashion (Hsu et al.,

1984). Furthermore, when antibody 2A4 was added to cultures of 3T3 cells in the absence of any exogenous 13 kD subunit, there was an enhancement of DNA synthesis in 3T3 cells, suggesting that antibody 2A4 can neutralize this subunit's activity as well as reverse the effect of density-dependent growth inhibition. It was not determined, however, whether the 13 kD subunit of the FGR-s was active independent of the presence of other polypeptides (Hsu et al., 1984).

Surprisingly, even though the monoclonal antibody successfully depleted the activity of FGR-s, the investigators did not utilize antibody chromatography as a purification system for the FGR-s. In order to further investigate which peptide was responsible for the biological activity, additional purification of the FGR-s was necessary. Based on isoelectric focusing analysis of the FGR-s, it was found that the 13 kD subunit had a pI of approximately 10.0 and the 10 kD subunit had a approximate pI of 7.5. The high pI of the 13 kD subunit suggested ion-exchange chromatography as a preparative purification procedure. DEAE-cellulose chromatography of [^{35}S] methionine-labeled preparation of FGR-s resulted in the separation of several components. When the fractions eluting from the ion-exchange column were assayed for growth inhibitory activity, only one component exhibited measurable activity. The sum of the growth activity in this component accounted for 80% of the total activity applied to the column. Hsu et al. (1984) found that this purification procedure results in a six-fold enrichment in terms of specific activity. Poly-acrylamide gel electrophoresis (PAGE) analysis of the inhibitory component revealed that this component was a single polypeptide, migrating at a position corresponding to a molecular weight of 13 kD. The migration of the molecule remained unchanged under reducing conditions (Hsu et al., 1984).

Despite purification to apparent homogeneity, further information concerning the nature of FGR-s is lacking. For instance, it is of greatimportance to learn if a

growth inhibitor produced by 3T3 cells would also inhibit the growth of other cell types, especially transformed cells. Furthermore, the monoclonal antibody could have been utilized as a powerful tool to determine whether or not other cells or tissues produce growth inhibitors with similar epitopes. Interestingly, Wells and Mallucci (1983), introduced a growth inhibitory molecule released into the medium by secondary cultures of mouse embryo fibroblasts. The composition and biophysical behavior of this polypeptide closely paralleled those of the FGR-s. Unfortunately, there was no collaborative work between the two groups and it is not known whether or not the monoclonal antibody against the FGR-s reacted with the inhibitory molecule isolated by Wells and Mallucci (1983). Since further investigation on FGR-s apparently has been discontinued, studies concerning the mechanism of the action of this inhibitor also are not available. For example, it is not clear what effects, if any, the FGR-s has on the events concerning cell growth regulation, effects such as changes in cytoplasmic pH, ion fluxes and or changes in the expression of growth related genes.

MAMMARY DRIVED GROWTH INHIBITOR (MDGI):

Lehman et al. (1983) isolated MDGI, a growth inhibitory molecule, from normal lactating bovine mammary glands. The MDGI was subsequently purified and characterized. Upon purification, the MDGI was shown to have a molecular weight of 13 kD. Based on the isoelectric focusing analysis, it was determined that the inhibitor had a pI of approximately 5.0 (Lehman et al., 1983).

MDGI was shown to inhibit the resumption of stationary Ehlrich ascites mammary carcinoma (EAC) cells in vitro, however, EAC cells from the exponential phase of growth were not inhibited (Lehman et al., 1983). Whether or not other cell types are inhibited by MDGI has not yet been determined. However, mouse antisera was raised against the 13 kD protein in order to determine if the 13kD peptide was actually responsible for total inhibitory activity. The antisera was utilized in a neutralization assay which demonstrated that the inhibitory activity of MDGI was in fact removed by the specific antiserum (Bohmer et al., 1985).

Tissue distribution of MDGI was analyzed by the use of an enzyme linked immunosorbent assay (ELISA). Among tissue supernatants from bovine mammary gland in different functional states, only lactating glands showed a positive reaction with the antiserum. However, samples from glands of a pregnant animal, newborn calves, calves and fetuses did not reveal the presence of reactive MDGI (Bohmer et al., 1985). Lung tissue supernatants have also shown a positive reaction on ELISA assay performed using anti-MDGI antisera (Bohmer et al., 1985). Even though these studies outlined the tissue distribution of MDGI, there is no explanation for the presence of the molecule in some tissues and not in others.

Further investigations revealed that both EGF and insulin are able to abolish the inhibitory effect of the MDGI. Likewise, the inhibitory activity is abolished when cells are preincubated for 4 h with serum before addition of the inhibitor. This

strongly suggested that the inhibition is exerted by a specific interaction of the inhibitory protein with the cells, however, data on specific binding of the protein to the target cells are lacking.

It stands to reason that growth inhibitors may also have modulated expression of certain genes since mitogens and growth factors modulate the expression of growth related genes. In order to determine whether or not modulation of gene expression is a pathway by which MDGI exerts its inhibitory activity, the effect of the MDGI on three of the oncogenes which are thought to be involved in growth regulation was studied by Lehman et al. (1987). In these studies, serum-induced expression of c-fos, c-myc, and c-ras in stationary cells was shown to be inhibited by the MDGI. However, in rapidly proliferating cells, MDGI did not affect oncogene induction. This provided evidence that, in a limited manner the MDGI may utilize growth inhibitory pathways which involve the modulation of expression of certain genes .

MDGI, similar to FGR-s, does not seem to have a wide host range, and whether or not its action is reversible is not known. Similarly, other studies concerning mechanism(s) of action of MDGI, such as receptor binding kinetics, tissue distribution and modulation of the expression of other oncogenes, are still incomplete. Interestingly, Bohmer et al. (1987) discovered that antibodies prepared against MDGI cross-reacts with FGR-s. This suggested that FGR-s and MDGI either have similar epitopes with which the antibody reacts or perhaps they are actually the same molecule.

TRANSFORMING GROWTH FACTOR-β (TGF-β)

Another growth inhibitor which is extremely interesting yet ambiguous with regards to its biological action is TGF-β. TGF-β is associated with a group of growth factors isolated from murine-sarcoma virus transformed 3T3 cells, designated transforming growth factors (TGFs). TGFs are hormonally active polypeptides functionally described as polypeptides that stimulate anchorage-dependent cells to grow in soft agar and have been detected in neoplastic and non-neoplastic cells in culture, as well as in tissues in vivo (Roberts et al., 1983). The two distinct TGFs which have been purified to apparent homogeneity are TGF-$\propto$ and TGF-β. TGF-$\propto$ is a powerful mitogen (Tadaro et al., 1978; Marquardt et al., 1983, 1984). While TGF-β is multifunctional, it has either stimulatory or inhibitory effects on proliferation, depending on which target cells are used (Roberts et al., 1985).

TGF-$\propto$ is a 5.6 kD, single-chain, acidic and heat stable molecule that has significant homology with epidermal growth factor (EGF) (Marquardt et al., 1984). This molecule mediates its biological activity through binding to the EGF receptor (Todaro et al., 1978; Marquardt et al., 1983, 1984). However, several experiments have indicated quantitative differences between TGF-$\propto$ and EGF (Derynck, 1986).

On the other hand, TGF-β is a completely different molecule. It has a molecular weight of 25 kD and is an acid stable molecule composed of two apparently identical polypeptide chains linked by disulfide bonds (Derynck et al., 1985). TGF-β was originally isolated from transformed cells (Moses et al., 1981, Roberts et al., 1981) however, it has become evident that it is distributed in a wide variety of tissues (Goustin et al., 1986). Platelets, which are the source of TGF-β found in serum, were found to be a rich source for purification of TGF-β. TGF-β is released by cells in an inactive form and the mechanism by which the molecule becomes activated in vivo is not clear (Lawrence et al., 1985, Lyons et al., 1988).

As mentioned previously, TGF-β has been defined as a multifunctional regulator of cell growth and differentiation (Roberts, 1985). It was originally identified by its capacity to reversibly induce anchorage independent growth of fibroblast cell lines (Childs et al. 1982). Later the growth inhibitory effects of TGF-β were described by its similarities to the structure and activity of BSC-1 growth inhibitor (GI), (Tucker et al. 1984). Heine et al. (1987) have further reported the involvement of TGF-β in wound healing. The biological activity of TGF-β is highly, variable depending on the cell line and/or the culture conditions (Moses et al., 1985). The opposing biological activity of TGF-β, however, has not been fully explained and the resolution of its biphasic potential may reveal important information related to both the positive and negative regulation of cell growth.

TGF-β has since been shown to be a potent inhibitor of a wide range of cells, in particular the epithelial cells and cells of the immune system (Sporn et al., 1986). Although anchorage-dependent proliferation of many normal epithelial cells is inhibited by TGF-β, many cells of fibroblast nature or transformed epithelial cells are resistant to its inhibitory effect (Masui et al., 1986; Shipley et al., 1986; McMahon et al., 1986; Knabbe et al., 1987). Even though many reports provide firm experimental data concerning the different cell types inhibited by
TGF-b, there are numerous controversies between different groups studying this peptide. For example, while some (Masui et al., 1986; Shipley et al., 1986; McMahon et al., 1986) have reported the resistance of transformed cells to the action of TGF-β, Goustin et al.(1986) reported the inhibition of a variety of transformed epithelial cell proliferation by this peptide.

It is quite disconcerting, however, that despite numerous studies concerning TGF-β, only one laboratory has reported on the reversibility of the inhibitory action of this peptide. Coffey et al. (1988a) used only one cell line, BALB/MK mouse

keratinocytes, as a model in order to show this reversible nature of TGF-β growth inhibitor.

There are specific cell surface receptors for TGF-β. These receptors are present on the surface of almost all cells tested including most normal and transformed fibroblasts, as well as epithelial and lymphoid cells of human, rat, or mouse origin (Tucker et al., 1984; Massague and Like 1985). However, there are three structurally different types of TGF-β receptors, and depending on the cell type and origin, only one of the three types of receptors may be present on the surface of any one cell type.

Surprisingly, a large number of biologically active molecules have shown homology with TGF-β. James and Bradshaw (1984) have previously categorized TGF-β with EGF due to structural similarities . However, TGF-β binds to specific cell surface receptors which are distinct from the EGF receptor (Associan et al., 1983; Frolik et al., 1983, 1984; Roberts et al., 1983; and Tucker et al., 1984). Interestingly, Tucker et al. (1984) demonstrated that TGF-β is similar to the BSC-1 growth inhibitor (GI) described by Holley et al. (1978). These two inhibitors, TGF-β (now referred to as TGF-β1) and GI (recently referred to as TGF-β2 [Hanks et al. 1988]), seem to have identical structure and biological activities. The native TGF-β2 molecule, similar to TGF-β1, is a peptide of 25 kD molecular weight. Both TGF-β1 and TGF-β2 stimulate the growth of AKR-2B, BSC-1 and CCL-64 (mink lung) cells and they bind to the same cell surface receptor. Furthermore, Bascom et al. (1988) reported as unpublished data that there is generally no qualitative difference in tissue, cell strain, or cell line distribution between TGF-β1 and TGF-β2. However, some opposing reports (Ohta et al., 1987; and Rosa et al., 1988) describe striking differences in the differential effects of TGF-β1 and TGF-β2. Experiments by Ohta et al. (1987) showed that TGF-β1, but not TGF-β2, is a potent inhibitor of hematopoietic progenitor cells. Furthermore, mesondermal induction studies by Rosa et al., (1988) on Xenopus laevis embryos

showed that TGF-β2, but not TGF-β1, was active in α-actin induction. TGF-β3 is another molecule with sequence homology to TGF-β1. Its structure and precursor has been deduced from cDNAs isolated from porcine and human cDNA libraries (Derynck et al. 1988). Further information on this peptide is lacking. Many other molecules have shown structural and sequence homology to TGF-β including: Inhibins (Mason et al., 1985), Mullerian inhibiting substance (Cate et al., 1986) and Drosophila decapentaplegic gene complex (Padgett et al., 1987) etc. It seems that TGF-βs are a family of closely related molecules and their structure and function may depend on the species, tissues and/or cells from which they are isolated.

Coffey et al. (1988a) have reported that neither total RNA nor total protein synthesis is affected by TGf-β. This report further suggested that selective changes occur in BALB/MK cells following TGF-β treatment, and that these changes result in the inhibition of DNA synthesis and subsequent cell division. In another report, Coffey et al. (1988b) have shown that TGF-β selectively inhibits the EGF induced expression of c-myc and KC genes in BALB/MK cells. In contrast, the EGF induced expression of c-fos mRNA was shown to be unaffected while β-actin mRNA was slightly increased. The modulation of gene expression due to the addition of TGF-β seems to follow the same general pattern as the other processes involved with the molecule. The inhibition and/or stimulation of the expression of different genes depends upon the type of cell and the culture condition. For instance, Takehara et al. (1987) reported that EGF induced expression of c-myc, KC and JE in endothelial cells was decreased by the addition of TGF-β. In the same system, however, there was no effect on the EGF induced expression of c-fos. In contrast Chambard and Pouyssegur (1988) reported that α-thrombin induced expression of c-myc and c-fos in chinese hamster fibroblasts was reported to be further induced by the addition of TGF-β.

In addition Coffey et al. (1988b) reported that TGF-β markedly decreased c-myc and KC gene expression in rapidly growing BALB/MK cells and reduced the EGF induction of these genes in a quiescent population of cells. TGF-β in this same system had no effect on the expression of c-fos.

GLYCOPEPTIDE INHIBITORS

In our laboratory, we have isolated and purified inhibitory glycopeptides from mouse and bovine cerebral cortex intact cells unique from any other inhibitor purified to this date. Kinders et al. (1979) described the first glycopeptide inhibitor from mouse cerebral cortex cells which was isolated by the mild protease treatment of intact cells. This brain cell surface glycopeptide (BCSG), had a molecular weight of about 25-30kD. It bound to **Ulex europaeus** agglutinin suggesting the presence of fucose (Kinders et al., 1980). Furthermore, Kinders et al. (1979) have shown the inhibition of protein synthesis and subsequent cell division of BHK-21 (baby hamster kidney) cells by the BCSG. Inhibition assays were carried out in order to assess whether or not BCSG exhibited any specificity with regard to cell lines. While most cells appeared to be sensitive to the inhibitor, the degree of sensitivity varied among cell lines and some transformed cells appeared to be highly refractory (Kinders et al., 1979). Since interferons are glycoproteins with inhibitory activity, the possible relationship between mouse interferon and BCSG was studied by Johnson et al. (1981). Several lines of evidence from these experiments suggested that BCSG, although able to inhibit cell protein synthesis, was not related to mouse cell interferon (Johnson et al., 1981).

The first bovine glycopeptide was isolated by Kinders and Johnson (1982). This glycopeptide was somewhat similar to the mouse inhibitor in that it bound to **Ulex europaeus** agglutinin, had a molecular weight of about 18 kD and a pI of 8.1. It also elicited inhibitory activity for BHK-21 cells in vitro, however, polyoma-virus-transformed BHK-21 cells were completely insensitive to the inhibitor (Kinders and Johnson, 1982). Furthermore, studies by Charp et al., (1983) showed that this glycopeptide arrested baby hamster kidney (BHK-21) cells and Chinese hamster ovary (CHO) cells in the G_2 phase of the cell cycle.

Sharifi et al. (1986a) have purified another glycopeptide from bovine brain cerebral cortex intact cells by mild protease treatment. This glycopeptide has a molecular weight of approximately 18 kD and a pI of 3.0. It bound to **Limulus polyhemus** agglutinin suggesting the presence of sialic acid. This sialoglycopeptide (SGP) was shown to be a potent inhibitor of protein, DNA and RNA synthesis in 3T3 cells.

Bascom et al. (1986) showed that there are specific cell surface receptors for the SGP on the surface of 3T3 cells and that binding of the SGP to the receptor was correlated with its biological inhibitory activity. This observation was compatible with a study that showed that SGP need not enter the target cell in order to elicit its action (Sharifi et al., 1986b).

We have recently purified the parent to the SGP from mouse and bovine brain whole cell homogenates. This parent molecule, which reacts with a polyclonal antibody preparation made against the 18 kD peptide, has a molecular weight of 70 kD. We have utilized the polyclonal antibody in Western blot assays in order to determine the tissue distribution of this glycoprotein. To date, every mouse tissue tested has shown reaction with the antibody, suggesting that at least one or more of the epitopes of the SGP is ubiquitous (Toole-Simms et al., unpublished data).

The SGP inhibitor also was shown to be capable of antagonizing the action of several growth factors and mitogens such as, EGF (Bascom et al., 1987), tumor promoter TPA (12-O-tetradecanoylphorbol-13-acetate)(Chou et al., 1987) and bombesin (Johnson and Sharifi, 1989).

Several other parameters that are associated with cell proliferation recently have been examined. These include changes in intracellular pH and Ca^{++} concentration that are altered in response to mitogens binding to their respective cell receptors. Since mitogens stimulate this cascade it stands to reason that growth

inhibitors may block events associated with these metabolic processes. In order to dissect the point in the cell cycle where the inhibitor may exert its activity, changes in intracellular pH and Ca^{++} were measured after treatment of cells with the SGP. The SGP alone caused a slight acidification of the cytoplasm and blocked the rise in pH induced by TPA or serum. In addition the SGP blocked the rise in intracellular Ca^{++} caused by mitogen stimulation (Toole-Simms and Johnson, unpublished data).

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CHAPTER II.
INHIBITION OF DNA SYNTHESIS AND CELL DIVISION BY A CELL SURFACE SIALOGLYCOPEPTIDE

ABSTRACT

We have isolated and purified a cell surface sialoglycopeptide (SGP) from bovine cerebral cortex cells that previously was shown to be a potent inhibitor of cellular protein synthesis. The following studies were carried out to characterize the potential ability of the SGP to inhibit DNA synthesis and to arrest cell division. Treatment of exponentially proliferating Swiss 3T3 cells with the SGP inhibitor resulted in a marked inhibition of thymidine incorporation within 24 h. When the SGP was removed from inhibited cultures, a sharp rise in ^{3}H-thymidine incorporation followed within 3 to 4 h that peaked well above that measured in exponentially growing cultures, suggesting that the inhibitory action of the SGP was reversible, and that a significant proportion of the arrested cells was synchronized in the mitotic cycle. In addition to DNA synthesis, the inhibitory action of the SGP was monitored by direct measurement of cell number. Consistent with the thymidine incorporation data, the SGP completely inhibited 3T3 cell division 20 h after its addition to exponentially growing cultures. Upon reversal there was a delay of 15 h before cell division resumed, when the arrested cells quickly doubled. Most, if not all, of the growth-arrested cells appeared to have been synchronized by the SGP. The SGP inhibited DNA synthesis in a surprisingly wide variety of target cells, and the relative degree of their sensitivity to the inhibitor was remarkably similar. Cells sensitive to the SGP ranged from vertebrate to invertebrate cells, fibroblast and epithelial-like cells, primary cells and established cell cultures, as well as a wide range of transformed cell lines.

INTRODUCTION

The regulation of mammalian cell proliferation involves a complex series of interactions that includes both growth stimulatory and growth inhibitory molecules. Inhibitors of cell proliferation have been identified from mouse and bovine brain (Kinders et al., 1980; Kinders and Johnson, 1982; Charp et al., 1983), liver (McMahon et al., 1982), platelets (Brown and Clemens, 1986), mammary gland (Bohmer et al., 1984; 1985), and from cell culture media (Holley et al., 1980, Hsu and Wang, 1986). The site and mechanism of action for most of these inhibitory molecules, however, often is unclear since most have not been purified to homogeneity.

Although growth factors have been extensively studied, information concerning the physical/chemical nature of growth inhibitors has been limited. Unlike growth factors, growth inhibitors, particularly those with significant hydrophobic domains that reside as cell surface molecules, have presented numerous problems regarding their purification and characterization. Furthermore, the necessity of detergents to maintain these hydrophobic components in solution or suspension has complicated the presentation of the putative regulatory molecules, in a physiologically relevant manner, to target cells. This has severely limited the measurement of their biological activity.

The isolation and purification to homogeneity of our SGP inhibitor obtained from intact bovine cerebral cortex cells, have been described previously (Sharifi et al., 1986a; Bascom et al., 1986). The SGP, which is a hydrophilic fragment of a larger glycoprotein (Sharifi et al., 1985), has a molecular weight of 18,000, a pI of 3.0, and the biological activity appears to reside in the polypeptide sequence of the molecule (Sharifi et al., 1986b). There are specific, high affinity receptors on the surface of 3T3 cells for the SGP, and the inhibition of protein synthesis is correlated with the occupancy of the receptor (Bascom et al., 1986).

In this report, we demonstrate that the SGP is a potent inhibitor of DNA synthesis and cell division, and that the inhibition is totally reversible. In addition, we demonstrate that the biological activity is equally effective against a wide variety of cells, both nontransformed and tumorigenic.

MATERIALS AND METHODS

Isolation and purification of the bovine SGP: The bovine SGP was purified as described previously (Sharifi et al., 1986a). Briefly, the SGP was released from intact bovine cerebral cortex cells by mild proteolysis, ethanol precipitated and extracted with chloroform/methanol (2:1, v/v). Following overnight dialysis against water, the SGP was lyophilized, and purified by DEAE ion-exchange chromatography, wheat germ agglutinin affinity chromatography and HPLC with a TSK 3000 column (Phenomenex, Rancho Palos Verdes, CA).

Serial dilutions of each preparation of the SGP inhibitor, contained in 40 μl of PBS buffer (10 mM Hepes, 120 mM KCl, 5 mM $MgCl_2$, pH 7.1), were tested for protein synthesis inhibition with 2 x 10^5 Swiss 3T3 cells in 100 μl of medium incubated with ^{35}S-methionine as described by Sharifi et al. (1986a). One unit of biological activity of the inhibitor was designated as the amount that provided a 25% reduction of protein synthesis.

Cell cultures : With the exception of HL-60 and PI-5.4 insect cells, established and primary cells were grown as monolayer cultures in a humidified incubator with 5% CO_2: 95% air atmosphere. All cells were plated (in 48 well plates from Costar, Cambridge, MA) and allowed to adhere to the cell culture vessel surface for 24 h prior to the addition of the SGP.

Human foreskin fibroblast cells (HSBP) and PC-12 cells (from Dr. Paul Sharp, Oakridge National Laboratories, TN.); Balb/c 3T3, Swiss 3T3 and NRK-52E cells (American Type Culture Collection, Rockville, MD); and, mouse fibrosarcoma cell lines 1316, 2337 and 2247 (from Dr. George W. Fortner, Kansas State University, Manhattan, KS) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories, Grand Island, NY) containing 10% calf serum. MD-BK cells (from Dr. Harish C. Minocha, Kansas State University, Manhattan KS) were grown in Ham's F-10 medium

(KC Biological, Lenexa, KS) plus 10% calf serum. BSC-1 (American Type Culture Collection, Rockville MD) were grown in Eagle's Minimum Essential Medium (MEM) (Flow Laboratories, McLean VA) plus 10% fetal calf serum.

The Plodia interpunctella insect cell line PI-5.4 (from Dr. Richard A. Consigli, Kansas State University, Manhattan, KS) was grown in suspension in Grace's Insect Media (Gibco Laboratories, Grand Island, NY)) plus 10% fetal calf serum. These insect cells were grown at room temperature and did not require incubation with CO_2. Chick embryo cells (CE) (from Dr. Richard A. Consigli, Kansas State University, Manhattan, KS) were grown in DMEM plus 10% calf serum. Primary mouse embryo and baby mouse kidney cells, (from Dr. Richard A. Consigli, Kansas State University, Manhattan KS) were grown in DMEM plus 10% fetal calf serum. HL-60 cells (from Dr. Melvin S. Center, Kansas State University, Manhattan KS) were grown in suspension in RPMI medium 1640 (Hazelton, Denver, CA) plus 10% fetal calf serum.

Measurement of DNA synthesis: DNA synthesis was measured by ^{3}H-thymidine incorporation essentially as described by Chou et al. (1986). Cells were seeded in 48-well tissue culture plates. The SGP was dissolved in PBS, added to the appropriate medium, filter-sterilized, and added to exponentially proliferating cultures. In all cases the final volume of medium added to cell cultures was 0.5 ml. Control cultures were incubated with the same medium, under the same conditions, but without the SGP inhibitor. After incubation for 20 h, 2.0 μCi of ^{3}H-thymidine (ICN Radiochemicals, Irvine, CA) was added to each well. Following a 2 h incubation period the cellular DNA was precipitated with ice-cold 10% trichloroacetic acid (TCA), the precipitates were washed three times with ice-cold TCA and then dissolved with a solution of 0.1N NaOH, 2% $NaCO_3$ and 1% SDS. The radioactivity in each sample was determined with a scintillation system.

HL60 transformed cells, which grow in suspension, were treated with the SGP as described above. However, after incubation of the cells with ^{3}H-thymidine for 2 h, the cells were transferred to glass tubes, precipitated with 10% ice-cold TCA and centrifuged at 1000 x g for 3 min. The precipitates were then washed 3 times with ice-cold TCA and finally dissolved in 1N NaOH.

In each case where the sensitivity of cells to the SGP was tested, Swiss 3T3 cells were incubated with and without the SGP as a relative control to compare cellular sensitivity to the inhibitor.

Determination of Cell Number: Swiss 3T3 cells were seeded in 48-well plates at 4.0 x 10^{3} cells/well. After a 4 h incubation period, the cells were fed with either 0.5 ml of complete medium alone, or with complete medium containing various concentrations of the SGP inhibitor. Cell numbers were determined after every generation time (20 h). Cells were detached from wells by trypsin and diluted 1:20 in Isoton II (Coulter Electronics Inc., Hialeah, FL.). The cell number was determined with a Coulter counter, model Zf, (Coulter Electronics Inc, Hialeah, FL.).

RESULTS:

Inhibition of Swiss 3T3 Cell DNA Synthesis

The potential influence of the SGP inhibitor on DNA synthesis was assessed with nonconfluent cultures of mouse Swiss 3T3 cells. The cells were incubated with either 0, 1, 2 or 4 units of the SGP for 20 h, and then 2.0 μCi of ^{3}H-thymidine added and the cultures were incubated for another 2 h. Compared to control cultures (no SGP), DNA synthesis was significantly inhibited and the inhibition was shown to be dose dependent (Fig. 1). The dose response of DNA synthesis was similar to that previously measured with 3T3 cell protein synthesis inhibition in that two units of protein synthesis inhibitory activity provided a 60% reduction of thymidine incorporation. In comparable protein synthesis inhibition assays with 3T3 cells and ^{35}S-methionine one unit of SGP provided 25% inhibition (Sharifi et al., 1986a; 1986b).

In order to determine if the inhibition of thymidine incorporation was reversible Swiss 3T3 cells were plated at a density of 2 x 10^{4} cells/cm^{2} and one unit of SGP was then added. After 22 h of incubation the media on all cultures were replaced with either complete medium containing 2.5% calf serum or with the same medium and serum that contained one unit of SGP. While DNA synthesis in cultures that received medium with SGP continued to be inhibited, a sharp increase in ^{3}H-thymidine incorporation was measured with cultures that received medium without the SGP (Fig. 2). The increase in DNA synthesis was initiated 3 to 4 h following the removal of the SGP, and the peak of ^{3}H-thymidine incorporation 10 to 12 h later even surpassed that measured in exponentially growing cultures (Fig. 2). The magnitude and kinetics of Swiss 3T3 cell recovery clearly indicated that the inhibitory affects of the SGP were reversible, and suggested that a significant fraction of the cell population was synchronized by the inhibitor.

Cell Growth Inhibition

Although the above data suggested that the SGP inhibited Swiss 3T3 cell DNA synthesis in a reversible manner, we reasoned that a more direct measurement of the growth inhibitory activity of the SGP would involve a direct assessment of its influence on cell division. In comparison to exponentially growing control cultures of Swiss 3T3 cells, cultures that received 4 units of SGP were markedly reduced in cell number, and by 20 h after the addition of the inhibitor cell division ceased (Fig. 3). Upon removal of the SGP from the cultures and after 12 to 15 h of constant cell number, however, the cells rapidly doubled (Fig. 3). The kinetics of reversal indicated that the action of the SGP was totally reversible and synchronized in the cell cycle.

Taking into account the 3 to 4 h delay in DNA synthesis following removal of the inhibitor, and the 12 to 15 h lag period prior to mitosis, the arrest point mediated by the SGP most likely was associated with the G1 phase of the cell cycle.

Target Cell Range of the SGP

A wide range of cell cultures were assayed with the SGP in order to investigate the potential range of target cell sensitivity to the inhibitor. Exponentially growing cultures were tested with various concentrations of the SGP as described in Fig. 1, and in each case the SGP was assayed in parallel with Swiss 3T3 cells as a comparative control. Surprisingly, a dose dependent inhibition of DNA synthesis was observed and all cell cultures tested were as sensitive to the inhibitor as the Swiss 3T3 cells. We discovered that DNA synthesis of cells obtained from a wide range of vertebrate species, including, human, mouse, and chicken, and even an established line of invertebrate cells were inhibited by the SGP inhibitor (Table 1). Furthermore, the SGP inhibitor was equally effective with epithelial and fibroblast cultures (Table 1).

Since all cell lines tested in Table 1, with the exception of primary chicken embryo cells, were established cell lines, we considered it important to extend our

studies to newly-established or primary cell cultures, from various origins, and transformed cell lines that generally are not sensitive to density-dependent growth inhibition. In each case the SGP was titrated and exponentially growing cultures were treated. The SGP was tested with 3T3 cells in parallel as a comparative control. DNA synthesis in a wide variety of primary and transformed cell cultures was found to be sensitive to the SGP. The sensitivity of the various cultures, as measured with increasing concentrations of SGP, was comparable to that measured with Swiss 3T3 cells (Table 2).

DISCUSSION:

In previous studies we have shown that the bovine SGP is a potent inhibitor of protein synthesis (Sharifi et al., 1986a), that the inhibitor does not have to be internalized to mediate its biological activity (Sharifi et al., 1986c) and that the SGP binds in a saturable manner to a specific cell surface receptor (Bascom et al., 1986). The surface receptor on Swiss 3T3 cells has been shown to be distinct from those mediating the binding of epidermal growth factor (Bascom et al., 1987), the tumor-promoting phorbol ester TPA (Chou et al., 1987), and the mitogen bombesin (Sharifi and Johnson, in preparation).

In the present study we have shown that the SGP also is a potent inhibitor of cellular DNA synthesis and that the inhibition is dose dependent (Fig. 1). An interesting feature of the biological action of the SGP is its totally reversible inhibitory activity on Swiss 3T3 target cells. By directly comparing cell numbers in exponentially dividing Swiss 3T3 cultures and populations treated with the SGP, we were able to show that the inhibitor arrested cells in the mitotic cycle and that the cell population was subsequently synchronized. Unlike another cell surface glycopeptide inhibitor that previously was shown to arrest cells in G_2 (Charp et al., 1983), the SGP kinetics of cell recovery, as measured by thymidine incorporation and cell division (Figs. 2 and 3), illustrated that the glycopeptide most likely inhibits the Swiss 3T3 cell cycle in or near G_1. Although several inhibitors of cell division have been under intense investigation, in most cases the site(s) of cell cycle arrest is not known. Lin et al., (1987) and Chambard et al., (1988) have shown that β-TGF arrests cells in two different phases of the cell cycle, the G_0/early G_1 and the G_1/S border while no single stage of the cell cycle appears to be uniquely sensitive to interferon action (Clemens and McNurlan, 1985).

The kinetics of cell recovery following the removal of the SGP revealed that the cell population was synchronized in the cell cycle (Fig. 3). The use of the SGP in

studies of cell cycling may have a valuable application since equivocal results often are obtained with other methods used by investigators to synchronize cell populations, i.e. ionizing and non-ionizing radiation, serum and nutrient depletion, and chemical agents like thymidine and hydroxyurea (Hohmann et al., 1975; Harrington et al., 1980). These and other agents frequently lead to poorly synchronized cell populations, altered cell metabolism and structure, and/or the death of a significant portion of the cells (Natruj and Datta, 1978; Pardee et al., 1978; Maurer-Schulitz et al., 1988).

A vast array of cells were sensitive to the inhibitory effects of the bovine SGP. Unlike a glycopeptide we previously isolated and purified from mouse brain cortex (Kinders et al., 1980) and a fucosylated glycopeptide obtained from bovine cerebral cortex (Kinders and Johnson, 1982), the SGP equally inhibited DNA synthesis in a wide variety of both nontransformed and transformed cells (Tables 1 and 2). In fact, sensitive cells included epithelial-like cells and fibroblast cells from a broad spectrum of vertebrate and invertebrate species. Of particular note was the sensitivity of the HL-60 and the PI-5.4 insect cells that grow in suspension rather than by firmly adhering to the culture vessel surface. Similar to protein synthesis inhibition by the SGP (Sharifi et al., 1986a; 1986b), decreased thymidine incorporation, mediated by the inhibitor, was independent of cellular attachment.

Although the target cell range of most naturally occurring inhibitors has not been reported, such a wide range of sensitive cells is quite unusual. Cell membranes prepared from senescent human diploid fibroblasts inhibit entry of replicating fibroblasts into the S phase of the cell cycle, although these membranes were unable to inhibit DNA synthesis in simian virus 40-transformed fibroblasts (Stein and Atkins, 1986). β-TGF seems to be a more effective inhibitor of epithelial cells while fibroblasts appear to be relatively refractory to its inhibitory action (Lin et al., 1987; Jetten et al., 1986; Baird and Durkin, 1986; Nakamura et al., 1985). However, Roberts et al. (1985)

reported that β-TGF inhibits the anchorage-dependent growth of NRK-49F cells as monolayers and also inhibits anchorage-independent growth of many, but not all, tumor cell lines tested. Interferons can inhibit the proliferative activity of at least some fibroblast and epithelial cells (Balkwill et al., 1978), and a number of studies have attempted to determine the relative sensitivity of normal versus tumor cells to the antiproliferative effects of interferons. Cultured human B cells and T cells proved resistant to high concentrations of human interferon, while DNA synthesis of myeloma cell lines, Burkitt's lymphoma and leukemic T cells were sensitive (Attallah et al., 1980).

The sensitivity of such a wide range of nontransformed and transformed cells to the bovine SGP inhibitor suggests that a functional receptor for the SGP is a common characteristic. However, the ability of transformed or tumorigenic cells to circumvent density-dependent growth inhibition may be based on their possessing little, if any, inhibitor of the SGP class, or that they release growth factors in sufficient quantity to maintain stimulated growth.

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Figure 1. Inhibition of DNA synthesis by the bovine sialoglycopeptide (SGP). Swiss 3T3 cells were plated as described in Materials and Methods. After a 24 h attachment period cells were treated with various concentrations of the SGP for 20 h. Cells then received 2.0 μCi of ^{3}H-thymidine per well, and were incubated for an additional 2 h. Following this incubation period, cellular DNA was precipitated and dissolved with 0.1N NaOH as described in the Materials and Methods section. The radioactivity was then measured by scintillation counting. The data were plotted as percent of thymidine incorporation in the presence of various concentration of SGP compared to control cultures that did not receive inhibitor. The data represent the mean $\pm$ SD of eight determinations.

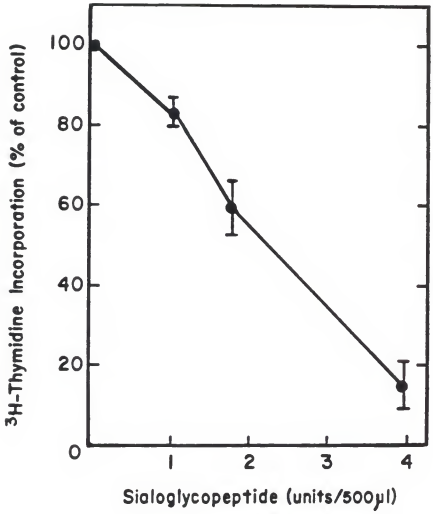
100
80
60
40
20
0
1
2
3
4
^{3}H-Thymidine Incorporation (% of control)
Sialoglycopeptide (units/500µl)

Figure 2. Reversal of the inhibitory effect of the sialoglycopeptide (SGP). Cells were treated as in Fig. 1 except following the 22 h incubation with 1 unit of the SGP (indicated by the arrow), one set of cultures received fresh medium without the SGP (open circles) while a second set received fresh medium containing 1 unit of SGP (closed circles). The broken line denotes the ^{3}H-thymidine incorporation in exponentially growing control cultures. The data represent the mean ± SD of triplicate determinations.

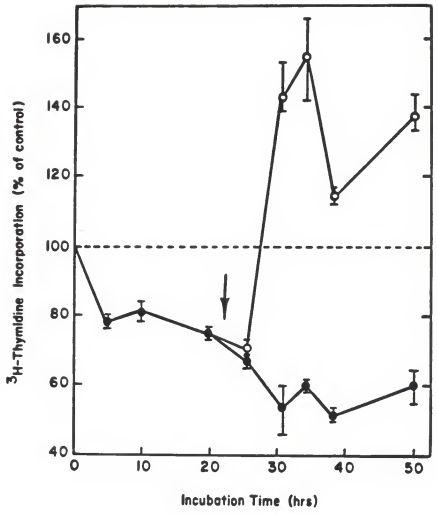
160
140
120
100
80
60
40
0
10
20
30
40
50
^{3}H-Thymidine Incorporation (% of control)
Incubation Time (hrs)

Figure 3. Inhibition of cell number by a sialoglycopeptide (SGP) and the reversal of its inhibitory effect on cell number. Cells were plated at a low seeding density (1.2×10^4), as described in the Materials and Methods section. Cells were allowed a 4 h attachment period and then one set was treated with 4 units of the SGP (first arrow). After a 20 h incubation period media were removed from all cultures and replaced with fresh medium without SGP (second arrow). Control cultures not treated with the SGP (closed circles), SGP inhibited and reversed cultures (open circles).

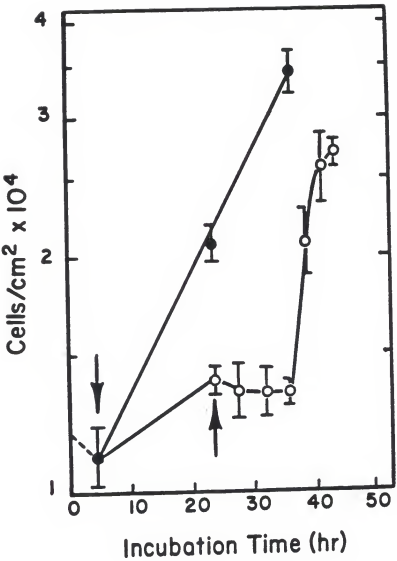
Cells/cm² x 10⁴
4
3
2
1
0
10
20
30
40
50
Incubation Time (hr)

Table 1. Target Cell Range of the Bovine SGP.

Cell Culture	Species	Cell-Type
HSBP	human	fibroblast
MD-BK	bovine	epithelial-like
Balb/c 3T3	mouse	fibroblast
Swiss 3T3	mouse	fibroblast
NRK-52E	rat	epithelial-like
BSC-1	monkey	epithelial-like
CE	chicken	fibroblast
PI-5.4	insect	(Indian meal moth, embryo)

Exponentially growing cultures were treated with various concentrations of SGP as described in Fig. 1 and thymidine incorporation was measured as described in Materials and Methods. Swiss 3T3 cells, with and without SGP in their medium, always servrd as comparative controls. All of the cells listed above were as sensitive to the SGP as the control Swiss 3T3 cells.

Table 2. Inhibition of Primary and Transformed Cell DNA Synthesis

Culture	Species	Cell-Type
kidney	mouse	primary
embryo	mouse	primary
HL-60	human	transformed (leukemia)
PC-12	rat	transformed (pheochromocytoma)
N-18	mouse	transformed (neuroblastoma)
IMR-32	human	transformed (neuroblastoma)
N2a	mouse	transformed (neuroblastoma)
1316	mouse	transformed (fibrosarcoma)
2247	mouse	transformed (fibrosarcoma)
2337	mouse	transformed (fibrosarcoma)

Swiss 3T3, primary and transformed cells were plated and treated with the sialoglycopeptide $(SGP)_2$ as described in Materials and Methods. After 20 h incubation with the SGP, percent ^{3}H-thymidine incorporation in all cell lines was compared to that of the Swiss 3T3 cell which served as controls. The inhibition of ^{3}H-thymidine incorporation in all cell cultures listed above was equal to the inhibition measured with the Swiss 3T3 cells.

CHAPTER III.
MODULATION OF GROWTH-RELATED GENE EXPRESSION AND CELL CYCLE SYNCHRONIZATION BY A SIALOGLYCOPEPTIDE INHIBITOR

ABSTRACT

When a cell surface sialoglycopeptide (SGP), isolated from intact bovine cerebral cortex cells, was incubated with exponentially growing Swiss 3T3 cells, cell proliferation was efficiently arrested. The inhibition was totally reversible since after removal of the SGP the arrested cells resumed their progress in the cell cycle in a synchronized manner for at least two divisions. Readdition of the SGP 4 h after reversal of the inhibition did not, however, affect the commitment of the cells to advance through metaphase, although progress through the cell cycle was once again inhibited after the cells re-entered the G_1 phase. The efficient nature of the SGP-mediated cell cycle arrest in G_1 provided us with a basis to examine potential changes in the expression of several genes that have been implicated in the early events associated with cell cycle progression. Upon serum stimulation of quiescent Swiss 3T3 cells, the induction of c-myc and c-fos expression were not influenced by the SGP at concentrations highly inhibitory to cell cycling. Expression of JE also was induced by serum, and the presence of the SGP had little effect on the expression of this growth related gene. KC expression was not appreciably stimulated by serum although, surprisingly, the addition of the SGP resulted in a significant increase in expression.

INTRODUCTION

The mechanism(s) responsible for the regulation of cell division is of central importance to numerous areas of cell biology ranging from developmental biology to immunology and to tumorigenesis. There is little question that proliferation of normal and tumorigenic cells is a result of interactions between both positive signals (growth factors) and negative signals (growth inhibitors). Although a considerable amount of information is available concerning the structure and biological action of growth stimulators, there remains little information on the interaction of naturally occurring growth inhibitors with regard to early events essential to the transition of cells from the G_O/G_1 phase of the cell cycle (Lau and Nathans, 1985).

The interaction of growth factors and tumor promoters with their specific cell surface receptors initiates a cascade of intracellular events that includes a transient increase in cytosolic pH, mobilization of Ca^{2+}, phosphoinositide metabolism, and protein phosphorylation (Epel and Dubè, 1987; Hesketh et al., 1987). A coordinate induction of the expression of genes associated with cell proliferation accompanies these metabolic events, and it is thought that the products of these genes play a central role in the initiation and/or maintenance of the proliferative state.

In some cases, the expression of these growth-related genes has been altered by the presence of growth inhibitory levels of transforming growth factor and tumor necrosis factor (Krönke et al., 1987; Fernandez-Pol et al., 1987; Takehara et al., 1987; Coffey et al., 1987). Whether these observations can be generalized to other cell lines and growth inhibitors isn't known. We previously have described an 18 kD cell surface sialoglycopeptide (SGP) inhibitor that was isolated from bovine cerebral cortex intact cells (Sharifi et al., 1986). This SGP has been purified to homogeneity, and is a potent inhibitor of cellular DNA synthesis and cell division (Fattaey et al., 1989). Its inhibitory action is reversible and nontoxic, cell cycle arrest occurs in G_O/G_1, and the biological

activity is directed against a wide array of eukaryotic cell species and types (Fattaey et al., 1989). The ability of the SGP to inhibit cell proliferation provided us with a rationale to investigate the potential influence of the inhibitor on serum-induced gene expression.

MATERIALS AND METHODS

Purification of the bovine SGP.

The bovine SGP was purified as described previously (Sharifi et al., 1986a). Briefly, the SGP was released from intact bovine cerebral cortex cells by mild proteolysis, concentrated by ethanol precipitation, and extracted with chloroform/methanol (2:1, v/v). Following overnight dialysis against water, the SGP was lyophilized, resuspended in 0.05 M sodium acetate (pH 5.5), and bound to a DEAE ion-exchange column. The unbound material was discarded and the bound proteins were eluted with 0.4 M NaCl in 0.05 M sodium acetate (pH 5.5). The eluted material was then concentrated, resuspended in PBS, and further purified by wheat germ agglutinin affinity chromatography. The unbound fraction was vacuum dried, resuspended in 0.1 M sodium phosphate buffer (pH 6.8), and purified by HPLC with a TSK 3000 SW size-exclusion column (Phenomenex, Rancho Palos Verdes, CA). The final preparation was dialyzed against dilute PBS, vacuum dried, and stored at -100C.

DNA synthesis inhibition was tested with purified SGP inhibitor resuspended in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% calf serum, sterilized through a 0.22 μ filter, and serially diluted in culture medium. 250 μl were incubated with exponentially growing Swiss 3T3 cell cultures for 20 h. DNA synthesis then was measured by incubating each culture with 2.0 μCi/ml of [^{3}H]thymidine for 1 h and then precipitating the cellular DNA with ice-cold trichloroacetic acid as described by Fattaey et al. (1989). One unit of biological activity of the inhibitor was designated as the amount that provided a 25% reduction of DNA synthesis ($\sim 3 \times 10^{-8}$ M).

Cell culture.

All Swiss 3T3 cells were grown as monolayer cultures in a humidified incubator with 5% CO_2: 95% air atmosphere. Cells were maintained in 250 μl of DMEM contain-

ing 10% calf serum and used during exponential growth for DNA synthesis and cell cycle arrest, and as confluent and quiescent cultures for serum stimulation.

Inhibition of Swiss 3T3 cell division.

Swiss 3T3 cells were seeded in 48-well plates at 4.0 X 10^3 cells/well. After a 4 or 10 h incubation period, the medium was removed and replaced with either 250 μl of complete medium alone, or with complete medium containing 2 units of the SGP inhibitor. After every generation time (20 h) the cell number was determined by detaching the cells from culture wells with trypsin and diluting (1:20) in Isoton II (Coulter Electronics Inc., Hialeah, FL). Cell numbers were measured with a Coulter Counter, model ZM, (Coulter Electronics Inc, Hialeah, FL.). After two generation times, the medium was removed from all cultures, the cells were washed two times with medium, fresh medium was added and the cell number was determined as described in specific experiments.

Cell culture for poly (A)$^+$ RNA isolation.

Swiss 3T3 cells were cultured in 75 cm^2 Costar flasks (Costar, Cambridge, MA), as described above, and grown for 5 days to quiescence. The culture medium was then removed and the cells were washed with 10 ml of serum-free DMEM. Serum-free DMEM media was then added and the cultures were reincubated for 48 h. Following this 48 h incubation, serum-free media was removed from the cells and replaced with DMEM containing 10% calf serum and various concentrations of the SGP. Several types of control cultures were used in these experiments. Control cultures, used to measure gene expression in quiescent cultures, either received conditioned DMEM medium that previously had been incubated with confluent Swiss 3T3 cell monolayers for 4 to 5 days or serum-free DMEM. To measure serum-induced expression, another set of cultures received fresh DMEM with 10% serum, while at the same time exper-

imental cultures received fresh DMEM with 10% serum and various concentrations of the SGP.

RNA analysis by Northern hybridization.

Northern hybridization was carried out with RNA that was extracted from all cell cultures using the method of Schwab et al. (1983) after 30 and 60 minutes incubation with 10% calf serum, with or without the presence of the sialoglycopeptide. Poly(A)$^+$ RNA was purified by oligo(dT) chromatography, and the mRNA was separated by electrophoresis in a 1.2% agarose-formaldehyde gel. Northern blotting was performed as previously described (Thomas, 1980). Purified cDNA inserts of c-myc (4.7 kb Bam H1-Xba1 insert), c-fos (1.1 kb Pst 1 insert), KC (0.82 kb Pst 1 insert), JE (0.75 kb Pst 1 insert), and B15 (0.68 kb Bam H1-Xba 1 insert), were labeled by random primer extension (Taylor et al., 1976). Following hybridization, the blots were washed in 0.1 X SSC, 0.1% SDS at 42C and exposed to Kodak XAR-5 film using an intensifying screen.

For all of the Northern blot analysis, hybridization with a control 1B15 probe demonstrated equal loading of RNA in each lane.

RESULTS

Synchronization of mouse Swiss 3T3 cell division by the SGP.

The ability of the SGP inhibitor to synchronize cell populations was assessed with nonconfluent and exponentially dividing monolayer cultures of mouse Swiss 3T3 cells. The cells were incubated in 250 μl of medium with 2.0 inhibitory units (8.0 units/ml) of the SGP for a period of 40 h, and the cell number was determined at least every generation time (20 h). Compared to control cultures which were fed with complete medium without the SGP experimental cultures, treated with the SGP were inhibited from dividing within 20 h (Fig. 1).

After a 40 h incubation period, the medium on all cultures was replaced with complete medium containing 10% calf serum. There was no significant change in the rate of proliferation of control cultures which continued exponential growth, while the number of cells in the previously SGP-treated cultures doubled abruptly within 10 h after removal of the inhibitor (Fig. 1). Following this doubling, the cell number remained essentially constant for a period of 12-15 h, after which the number of cells, again, in a synchronous fashion completely doubled. The kinetics of recovery clearly indicated that the SGP-mediated cell cycle arrest was totally reversible, and that a single exposure of Swiss 3T3 cells to the SGP inhibitor synchronized the cell populations for at least two divisions.

The kinetics of inhibition and the synchronized recovery of the Swiss 3T3 cell populations, as shown in Fig. 1, suggested that a single arrest point was associated with the inhibitory action of the SGP. To determine if there was a single arrest point or multiple points in the cell cycle where the SGP might act, the inhibitor was used to synchronize Swiss 3T3 cell populations, and the inhibition again was reversed by feeding the cultures with complete medium without the SGP. In this series of experiments, 4 h after the SGP was removed, one set of the previously SGP-treated

cells received medium with 2.0 units of SGP while a second set received fresh medium without the inhibitor. Cells retreated with the SGP continued to traverse the cell cycle and divided, but again they were inhibited after the mitotic phase (Fig. 2). This illustrated that there was not a second arrest point between 4 hr after reversal and mitosis, and that the inhibition again occurred in the G1 phase of the cell cycle.

The efficient cell cycle arrest that was mediated by the SGP, and the synchronous nature of cell division following reversal of the inhibition, provided a basis to assess the relationship between gene expression and cell cycling. To determine the potential influence of the SGP on the expression of c-myc and c-fos, monolayers of Swiss 3T3 cells were grown for 5 days to confluence and the quiescent cultures were then fed with serum-free DMEM and reincubated for 48 hr.

The SGP does not inhibit serum induced expression of c-myc and c-fos.

The potential influence of SGP inhibitor on serum induced expression of c-myc and c-fos was assessed with confluent cultures of mouse Swiss 3T3 cells. After the 48 hr incubation period in serum-free medium, the medium was removed and replaced with either conditioned medium, serum-free medium, or complete DMEM containing 10% calf serum and various concentrations of the SGP inhibitor. Poly(A)$^+$ RNA was extracted 30 and 60 min after the various treatments and analyzed by Northern blot for both

c-myc and c-fos expression. Results of Northern blot analysis showed that c-fos expression was measurably induced by serum stimulation after 30 min and by 60 min the expression was significantly reduced. Even though highly inhibitory to cell division, the presence of 4 to 12 inhibitory units/ml of the SGP had little, if any, influence on c-fos expression (Fig. 3). Although slight, it appeared as if the expression of c-fos was stimulated at 60 min in the presence of 12 units/ml of the SGP. Unlike c-fos, the expression of c-myc was only minimal at 30 min with an approximately 30-fold

induction at 1 h after the addition of DMEM and 10% calf serum. The expression of c-myc, however, was not measurably affected, at 30 min or 60 min, by any of the concentrations of the SGP (Fig. 4).

Dose dependent induction of KC expression by the SGP.

The KC gene is a platelet-derived growth factor (PDGF) inducible gene that commonly is measured in cell growth regulation studies. To examine the influence of the SGP on the induced expression of KC, confluent and quiescent Swiss 3T3 cell cultures were incubated in serum-free DMEM for 48 hr, the medium was removed and then the cultures were fed with either conditioned medium, serum-free medium, or complete DMEM containing 10% calf serum and 0 to 12 units of the SGP inhibitor. Northern blot analysis of poly $(A)^+$ RNA, isolated 30 min after serum stimulation, showed only minimal KC expression under all experimental conditions. Surprisingly, an increase in KC gene expression was observed after 60 min incubation with the SGP (Fig. 5). In addition, the SGP-induced induction observed at 60 min was shown to be SGP dose dependent in that there was a measureable increase in KC expression with concentrations of the SGP from 4 to 12 units/ml.

Increased expression of JE in the presence of the SGP.

Expression of the PDGF inducible gene JE, which also has been correlated with cell cycling, was stimulated by the replacement of serum-free medium with DMEM containing calf serum. JE expression was measureable within 30 min of the addition of medium containing calf serum although the expression was significantly increased at 60 min. After 30 min of incubation with serum-containing DMEM, the presence of 4 and 12 units/ml of the SGP appeared to increase expression although there was a marked reduction with highest concentration of the inhibitor used, 12 units/ml. However, upon 60 min of incubation, when JE expression was markedly increased, the SGP enhanced the induction to levels above that measured with serum alone (Fig. 6).

DISCUSSION

The relationship between protooncogene expression and the commitment of quiescent cell populations to enter the cell cycle has been an intensive topic of study. In the absence of purified naturally occurring cell growth inhibitors, however, the important links between inhibitory signals and mitogenic cues (growth factors and tumor promoters), have been difficult to establish. To this end, we have isolated and purified a cell surface SGP that is a potent inhibitor of cellular protein synthesis, DNA synthesis, and cell division (Sharifi et al., 1986; Fattaey et al., 1989). The SGP is an effective inhibitor of the mitogenic action of 12-O-tetradecanoylphorbol-13-acetate (TPA) (Chou et al., 1987), epidermal growth factor (Bascom et al., 1987), and bombesin (Johnson and Sharifi, 1989). Unlike other reported inhibitors, the SGP has an unusually broad range of sensitive target cells including vertebrate and invertebrate cells, both nontransformed and transformed cells, and fibroblast and epithelial-like cells (Fattaey et al., 1989). Kinetic studies have shown that Swiss 3T3 cells, the subject of the present study, are arrested by the SGP in the G_0/G_1 phase of the mitotic cycle.

The inhibitory action of the SGP is nontoxic and totally reversible as shown by the doubling of the cell population several hours after the removal of medium containing the inhibitor (Fig. 1). Furthermore, the kinetics of reversal and the continued cell division showed that the cell population remained synchronized through at least two subsequent divisions (Fig. 1). Readdition of the SGP to Swiss 3T3 cell cultures 4 h after the reversal of cell cycle arrest did not abrogate their continuing through mitosis (Fig. 2). This illustrated that there was not a second arrest point, that was mediated by the SGP, between the original G_0/G_1 block and cell division.

The efficient and nontoxic nature of the SGP inhibition of Swiss 3T3 cycling offered an opportunity to access its ability to modulate expression of several genes that have been associated with early events leading to cell proliferation. When quiescent

Swiss 3T3 cell monolayer cultures were serum-stimulated, there was a marked induction of c-fos expression. C-fos is one of the earliest genes to be stimulated after the addition of growth factors (Kruijer et al., 1984; Prywes and Roeder, 1986), and the measured induction of c-fos expression within 30 min and the relatively short half-life of the transcripts (Fig. 3), were consistent with earlier reports by others (Kruijer et al., 1984; Denhardt et al., 1986; Mörike et al., 1988). The presence of the SGP at concentrations more than adequate to maintain cell cycle arrest (Fig. 1), did not diminish the measured levels of c-fos mRNA in the stimulated cells (Fig. 3). In addition, there was little influence by the SGP on the half-life of c-fos transcripts with the exception of a slight, but perceptible, sparing in the presence of 12 units/ml of the inhibitor.

Upon growth factor stimulation of confluent cell cultures the induction of c-myc follows c-fos (Greenberg and Ziff, 1984; Muller et al., 1984; Blanchard et al., 1985; Denhardt et al., 1986). Northern analyses in our experiments showed only a slight stimulation of c-myc expression at 30 min and a significant accumulation of c-myc transcripts at 60 min following the addition of serum (Fig. 4). Similar to the observations with the induced expression of c-fos, however, the presence of 4 to 12 units/ml of the SGP had no measured effect on the induction of c-myc expression.

Unlike c-fos and c-myc, expression of the KC gene, which has been shown to be activated in Swiss 3T3 cells within 60 min after the addition of PDGF (Cochran et al., 1983), was only slightly stimulated by the addition of serum to quiescent Swiss 3T3 cell monolayers. Interestingly, the expression of KC was stimulated 60 min after the addition of the SGP, and the relative increase of KC transcripts appeared to be dependent on the concentration of the inhibitor (Fig. 5). The expression of JE, another PDGF dependent gene associated with entry of the Swiss 3T3 cells into the mitotic cycle (Cochran et al., 1983), was induced by the addition of serum. Like the expression of KC, JE mRNA synthesis appeared to be stimulated by the presence of the SGP

inhibitor (Fig. 6). However, the relative amount of JE mRNA did not seem to be significantly increased by incremental concentrations of the SGP.

We considered the possibility that the SGP did not increase the transcription of the KC and JE genes but rather mediated a diminished rate of protein synthesis that decreased the levels of nuclease. An inhibition of nuclease activity could increase the half-lives of the transcripts thereby providing what would appear to be an increased synthesis of mRNA as measured by Northern analyses. A superinduction of c-fos has been reported with Swiss 3T3 cells treated with a combination of PDGF and cycloheximide (Cochran et al., 1984). It already is known that the protein synthesis inhibitory activity of the SGP can be measured within minutes of its addition to target cells (Sharifi et al., 1986; Bascom et al., 1986). This explanation does not seem plausible, however, since decreased nuclease activity would be expected to also have a sparing influence on c-fos and c-myc transcripts. It is clear that the typical transient appearance of c-fos transcripts was not affected by the presence of the SGP (Fig. 1), and the relative amount of c-myc transcripts appeared similar between cells incubated with calf serum alone and those cultures also incubated with 4 to 12 units/ml of the SGP (Fig. 2). Why KC and JE expression were stimulated by the presence of growth inhibitory levels of the SGP, and if this unusual observation is related in any fashion to cell growth control, remain to be resolved.

We conclude that the presence of the SGP clearly does not inhibit the expression, or diminish the number of transcripts, of these four genes that have been associated with a commitment of quiescent Swiss 3T3 cells to enter the cell cycle. Although previous observations have shown that the inhibitory action of the SGP is rapidly mediated when abolishing the action of growth factors and a phorbol ester tumor promoter (Chou et al., 1987; Bascom et al., 1987; Johnson and Sharifi, 1989), the inhibitory activity does not seem to be associated with a transcriptional block.

Since it is, however, the translation of these mRNA transcripts that actually leads to cell proliferation (Moelling, 1986; Rozengurt, 1986; Palmiter and Brinster, 1986), it is possible that the polypeptide products associated with these early inducible genes are not synthesized in the growth arrested cells. Alternatively, these data may disclose that the SGP-induced G_0/G_1 cell cycle arrest point (Fattaey et al., 1989) may be in mid- or late-G_1 where the inhibitor would not be expected to block the expression of immediate or early gene expression.

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Figure 1. Synchronization of Swiss 3T3 cells by the bovine sialoglycopeptide (SGP) inhibitor. Swiss 3T3 cells were plated and allowed to attach to the culture wells for 10 h, and then 2 units of inhibitor were added to cells (first arrow). After two generation times, the medium with the inhibitor was removed and fresh medium was added to all cultures (second arrow). The data represent the mean ± S.D. of triplicate cultures. Control cultures not treated with the SGP (closed circles); SGP inhibited and reversed cultures (open circles).

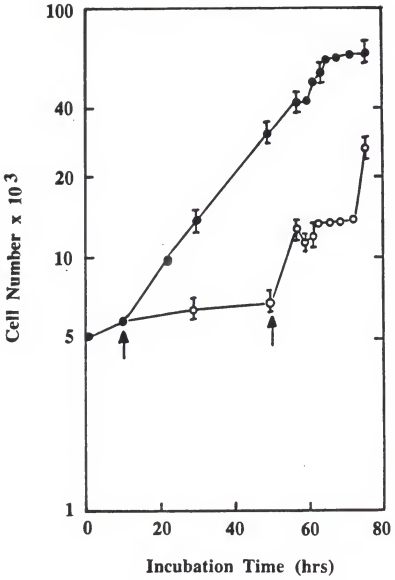
100
40
20
10
5
1
Cell Number x 10^3
0
20
40
60
80
Incubation Time (hrs)

Figure 2. Readdition of the SGP after reversal of the inhibition of cell cycling. Swiss 3T3 cells were plated and allowed to attach to culture plate for 10 h, 2 inhibitory units of SGP were then added (first closed arrow). After two generation times, the media from all cultures was removed and fresh media, without the SGP, was added (open arrow). 4 h following the removal of the SGP the cells previously inhibited by the SGP again received 2 units of the SGP (second closed arrow). The data represent the mean ± S.D. of triplicate cultures. Control cultures not treated with the SGP (closed circles); SGP inhibited, reversed, and reinhibited cultures (open circles).

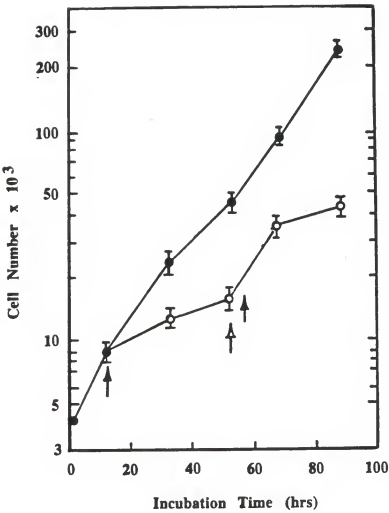
300
200
100
50
10
5
3
Cell Number x 10^3
0
20
40
60
80
100
Incubation Time (hrs)

Figure 3. Effect of the bovine sialoglycopeptide (SGP) on the expression of c-myc in serum-stimulated quiescent Swiss 3T3 cells. Swiss 3T3 cells were grown to quiescence and incubated with DMEM with 10% calf serum as described in the Materials and Methods. After 30 and 60 min, poly(A)$^+$ RNA was isolated and analyzed by Northern blots as described. Each lane contained 1.0 μg of poly(A)$^+$ RNA that was hybridized to a ^{32}P-labelled cDNA probe as described in the Materials and Methods.

c-myc

Conditioned Media
Serum Free
10% CaS + DMEM
+ 4 Units/ml
+ 8 Units/ml
+ 12 Units/ml
SGP
30'

Conditioned Media
Serum Free
10% CaS + DMEM
+ 4 Units/ml
+ 8 Units/ml
+ 12 Units/ml
SGP
60'

Figure 4. Effect of bovine sialoglycopeptide on expression of c-fos in serum-stimulated quiescent Swiss 3T3 cells. The experiment was carried out as described in Fig. 3. Each lane contained 1.0 μg of poly(A)$^+$ RNA that was hybridized to a ^{32}P-labelled cDNA probe as described in the Materials and Methods.

c-fos

Conditioned Media
Serum Free
10% CaS + DMEM
+ 4 Units/ml
+ 8 Units/ml
+ 12 Units/ml
SGP
30'

Conditioned Media
Serum Free
10% CaS + DMEM
+ 4 Units/ml
+ 8 Units/ml
+ 12 Units/ml
SGP
60'

Figure 5. Effect of bovine sialoglycopeptide (SGP) inhibitor on the expression of KC in serum-stimulated quiescent Swiss 3T3 cells. The experiment was carried out as described in Fig. 3. Each lane contained 1.0 μg of poly(A)$^+$ RNA that was hybridized to a ^{32}P-labelled cDNA probe as described in the Materials and Methods.

KC

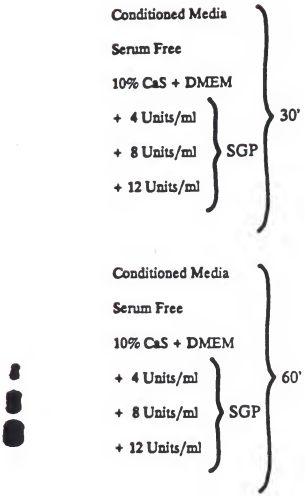
Conditioned Media
Serum Free
10% CaS + DMEM
+ 4 Units/ml
+ 8 Units/ml
+ 12 Units/ml
SGP
30'
Conditioned Media
Serum Free
10% CaS + DMEM
+ 4 Units/ml
+ 8 Units/ml
+ 12 Units/ml
SGP
60'

Figure 6. Effect of bovine sialoglycopeptide (SGP) inhibitor on the expression of JE in serum-stimulated quiescent Swiss 3T3 cells. The experiment was carried out as described in Fig. 3. Each lane contained 1.0 μg of poly(A)$^+$ RNA that was hybridized to a ^{32}P-labelled cDNA probe as described in the Materials and Methods.

JE

Conditioned Media
Serum Free
10% CaS + DMEM
+ 4 Units/ml
+ 8 Units/ml
+ 12 Units/ml
SGP
30'

Conditioned Media
Serum Free
10% CaS + DMEM
+ 4 Units/ml
+ 8 Units/ml
+ 12 Units/ml
SGP
60'

CHAPTER IV.
SUMMARY

SUMMARY

Cellular growth and homeostasis clearly are under tight restriction. This control is provided by the two major classes of substances, growth stimulatory molecules and growth inhibitory molecules. A loss of growth control, then, is prerogative to a deviation from the delicate equilibrium maintained by these molecules. The analysis of this regulatory balance is dependent upon the understanding of these contributing elements, growth factors and growth inhibitors. Many growth stimulatory molecules have been isolated, purified and studied extensively. On the other hand, only a limited number of growth inhibitors have been recognized. However due to the difficulties involved in the purification of these few inhibitors, their mechanism of action is still unknown.

Despite all obstacles involved in the purification of these molecules, we have isolated inhibitory glycopeptides from mouse and bovine brain cerebral cortex intact cells. Kinders et al. (1979) described a glycopeptide inhibitor from mouse brain isolated by mild protease treatment of intact cells. A similar molecule was later isolated from bovine brain cerebral cortex cells by Kinders et al. (1982). This inhibitor had a molecular weight of 18 kD, a pI of 8.1 and was shown to reversibly inhibit protein synthesis and division of a variety of normal cell types. However, transformed cells were either slightly sensitive or completely refractory to the inhibitors action. This glycopeptide was shown to inhibit baby hamster kidney (BHK-21) cells in the G_2 phase of the cell cycle (Charp, et al., 1983).

Another growth inhibitor was isolated from bovine brain cerebral cortex intact cells by Sharifi et al. (1986a). This was a sialidated peptide with a molecular weight of 18 kD, a pI of 3.0 and was designated sialoglycopeptide (SGP). The SGP was shown to inhibit protein and DNA synthesis in mouse 3T3 fibroblast cells (Sharifi, et al., 1986a; Chou, et al., 1986). There are specific high affinity receptors for the SGP on the

surface of 3T3 cells and binding of the molecule to cells is correlated with the inhibition of cellular protein synthesis (Bascom et al., 1986).

The purpose of the present work was to further characterize the mechanism of action of the SGP. In order to determine the potential range of target sensitivity to the inhibitor, a wide range of exponentially growing cell cultures were assayed with the SGP. In each case the SGP was assayed in parallel with Swiss 3T3 cells as a comparative control. Surprisingly, all cell cultures tested were as sensitive to the inhibitor as the Swiss 3T3 cells. We discovered that DNA synthesis of cells obtained from a wide range of vertebrate and non vertebrate species, epithelial and fibroblast cultures was inhibited by the SGP. In addition, a number of transformed cells DNA synthesis was also inhibited by the SGP. Furthermore, we discovered that actual cell division in Swiss 3T3 cells was blocked by the SGP and that the inhibitory action of the this molecule on Swiss 3T3 cells DNA synthesis and cell division was totally reversible, therefore non toxic to cells. We have shown the ability of the SGP to synchronize cell populations in non-confluent and exponentially dividing mouse Swiss 3T3 cells. A single exposure of cells to the SGP mediated synchronization of cell population for at least two divisions. The kinetics of inhibition and the synchronized recovery of the Swiss 3T3 cell populations suggested that the entire cell population was arrested in th G_1 phase of the cell cycle. We also described experiments that lead us to believe that there is a single arrest point in the cell cycle. Furthermore, to discover the early events that may be modulated by the SGP, we examined the effect of the SGP on the expression of several growth related genes. We have shown that the growth inhibition mediated by the SGP is not related to an inhibition of c-myc, c-fos, JE or KC gene expression. In fact, serum induced expression of KC and JE, two genes associated with cell division, appeared to be enhanced by the addition of the SGP. The expression of c-myc seemed not to be affected by the addition of the SGP while c-fos expression was

slightly enhanced with high concentration of the inhibitor after 60 minutes of treatment.

AN INVESTIGATION OF CELL CYCLE EVENTS MODULATED BY A SIALOGLYCOPEPTIDE INHIBITOR

by

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AN ABSTRACT OF A THESIS

submitted in partial fullfillment of the
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ABSTRACT

We have isolated and purified, to apparent homogeneity, a sialoglycopeptide (SGP) from bovine brain cerebral cortex cell surfaces. This glycopeptide has been shown to be a potent inhibitor of cellular protein synthesis. The studies we present in this dissertation were carried out to characterize the potential ability of the sialoglycopeptide to modulate several cell growth-related processes. Initially treatment of exponentially proliferating Swiss 3T3 cells with the SGP inhibitor resulted in a marked inhibition of thymidine incorporation within 24 h. When the SGP was removed from inhibited cultures, a sharp rise in ^{3}H-thymidine incorporation followed within 3-4 h, and peaked well above that measured in exponentially growing cultures. This suggested that the inhibitory action of the SGP was reversible and that a significant proportion of the arrested cells was synchronized in the mitotic cycle.

In addition to DNA synthesis, the inhibitory action of the SGP was monitored by direct measurement of cell number. Consistent with the data on thymidine incorporation, the SGP completely inhibited Swiss 3T3 cell division 20 h after its addition to exponentially growing cultures. Upon reversal there was a delay of 15 h before cell division resumed, when the arrested cells quickly doubled. Most, if not all, of the growth-arrested cells appeared to have been synchronized by the SGP.

The SGP inhibited DNA synthesis in a surprisingly wide variety of target cells, and the relative degree of their sensitivity to the inhibitor was remarkably similar. Cells sensitive to the SGP ranged from vertebrate to invertebrate cells, as well as a wide range of transformed cell lines. In another series of experiments the SGP was incubated with exponentially growing Swiss 3T3 cells and cell proliferation was totally stopped. The inhibition was again totally reversible since after removal of the SGP, the arrested cells resumed their progress in the cell cycle in a synchronized manner for at least two divisions. Readdition of the SGP 4 h after reversal of the inhibition did not,

however, affect the commitment of the cells to advance through mitosis, although progress through the cell cycle was once again inhibited after the cells entered G_1.

The efficient nature of the SGP-mediated cell cycle arrest in G_1. provided us with a basis to examine potential changes in the expression of several genes that have been implicated in the early events associated with cell division. Upon serum stimulation of quiescent Swiss 3T3 cells, the induction of c-myc and c-fos expression were not influenced by the SGP at concentrations highly inhibitory to cell cycling. Expression of JE also was induced by serum, and the presence of the SGP had little effect on the expression of this growth related gene. KC expression was not appreciably stimulated by serum although, surprisingly, the addition of the SGP resulted in a significant increase in expression.

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